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134

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(54) Title: OLIGONUCLEOTIDES SPECIFIC FOR THE <i>marORAB</i> OPERON			
(57) Abstract Disclosed are synthetic oligonucleotides complementary to a transcript of the <i>marORAB</i> operon which inhibit expression of a gene in the operon. Also disclosed are methods of reducing bacterial resistance to antibiotics, and pharmaceutical formulations containing <i>marORAB</i> -specific oligonucleotides of the invention.			

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OLIGONUCLEOTIDES SPECIFIC FOR THE *marORAB* OPERON

BACKGROUND OF THE INVENTION

This invention relates to the control of prokaryotic multidrug susceptibility.

5 More specifically, this invention relates to the use of oligonucleotides for the treatment of diseases, disorders, and conditions associated with drug resistance in bacteria.

Bacterial antibiotic and antimicrobial resistance has been recognized since the advent of antimicrobial agents. In the past, the appearance of resistant microorganisms has been addressed by the continued availability of effective alternative drugs. As
10 reported by Neu (*Science* (1992) **257**:1064-1073), the situation has recently changed dramatically, leading to increasing morbidity and mortality. The growing number of pathogens resistant to multiple, structurally unrelated drugs, and the fact that no new class of antimicrobials is likely to be introduced before the end of the decade, have been blamed for the present crisis of clinical and epidemiologic significance (see, e.g. Neu,
15 *supra*). Thus, as discussed extensively in the medical and scientific literature, there is a growing need to formulate effective therapeutic approaches to counter the emergence of novel bacterial strains resistant to antibiotics.

Resistance to an antimicrobial agent may be an inherent property of the infecting organism, or may result from mutation or from transfer of an extrachromosomal genetic
20 determinant, such as plasmids and transposons, followed by selection of resistant organisms. In recent years there has been increased interest in the role of chromosomal sequences involved in conferring antibiotic resistance. A novel chromosomal stress response locus, the multiple antibiotic resistance (*mar*) locus has been shown to control the expression of chromosomal genes involved in intrinsic multidrug
25 susceptibility/resistance to multiple, structurally different antibiotics and other noxious agents in *Escherichia coli* and in other members of the *Enterobacteriaceae* family (Cohen et al. (1988) *J. Bacteriol.* **170**:5416-5422; Cohen et al. (1993) *J. Infec. Dis.* **168**:484-488).

The *mar* locus has been reported to include two transcriptional units, *marC* and
30 *marRAB*. Each unit is divergently transcribed from a central regulatory region, *marO* (Cohen et al. (1993) *J. Bacteriol.* **175**:1484-1492; and Goldman, J. et al. (1996) *Antimicrobiol. Agents Chemo.* **40**:1266-1269). Both operons, *marORAB* and *marC* are necessary for the full expression of the Mar phenotype. Transcription of the *marORAB* operon is inducible two to three fold by tetracycline, chloramphenicol, salicylate, and
35 other structurally unrelated compounds (Cohen et al., *supra*). Activated cells become resistant not only to multiple unrelated antibiotics but also to oxidative stress agents and

- 2 -

organic solvents (Cohen et al., *supra*; George, et al. (1983) *J. Bacteriol.* 155:531-540; George et al. (1996) *FEMS Micro. Let.* 139:1-10).

In the presence of selective agents (e.g., tetracycline, chloramphenicol, nalidixic acid, rifampin, penicillin, and cephalosporin) Mar mutants arise spontaneously at a
5 frequency of 10^{-7} (George, et al., *J. Bacteriol. supra*). Such mutants have been reported to favor the accumulation of secondary mutations leading to the expression of higher levels of resistance to novel or improved antimicrobial agents. For example, Mar mutants have recently been found among fluoroquinolone-resistant clinical isolates of *E. coli* (Maneewannakul et al. (1996) *Antimicrob. Agents Chemo.* 38:542-546).

10 Characterization of several Mar mutant resistant strains has revealed constitutive transcription of mRNA from the *marORAB* operon as a result of various mutations within that operon (Cohen et al., *supra*). Consistent with these findings, the disruption of the *mar* locus has correlated with the complete loss of resistance. The resistance phenotype has been completely reversed to susceptibility by insertion of Tn5, a
15 transposon element, into the *marA* gene of the *E. coli* chromosome. (George et al. (1983) *J. Bacteriol.* 155:541-548).

A promising new approach to antimicrobial therapy lies in the use of short synthetic strands of nucleic acids, called antisense oligonucleotides, to control gene expression. Inhibition of gene expression by antisense oligonucleotides relies at least in
20 part, on the ability of the oligonucleotide to bind a complementary messenger RNA sequence, thereby preventing its translation (see generally, Agrawal (1992) *Trends in Biotech.* 10:152; Wagner et al. (1994) *Nature* 372:333-335; and Stein et al. (1993) *Science* 261:1004-1012). Synthetic oligonucleotides administered exogenously compose an alternate class of therapeutic agents and have been used successfully in both
25 prokaryotic and eucaryotic systems.

Antisense oligonucleotides have been developed as antiparasitic agents, although none have been demonstrated to reverse the drug resistant phenotype of a drug resistant parasite strain. PCT publication No. WO 93/13740 discloses the use of antisense oligonucleotides directed to nucleic acids encoding the dihydrofolate reductase-
30 thymidilate synthase gene of *P. falciparum* to inhibit propagation of drug-resistant malarial parasites. Rapaport et al. (*Proc. Natl. Acad. Sci. (USA)* (1992) 89:8577-8580) teaches inhibition of the growth of chloroquine-resistant and chloroquine-sensitive *P. falciparum* *in vitro* using oligonucleotides directed to the dihydrofolate reductase-thymidylate synthase gene. PCT publication No. WO 94/12643 discloses antisense
35 oligonucleotides directed to nucleic acids encoding a carbamoyl phosphate synthetase of *P. falciparum*. Tao et al. (*Antisense Res. Dev.* (1995) 5:123-129) teaches inhibition of propagation of a schistosome parasite by antisense oligonucleotides. Early experiments

- 3 -

by Jayaraman showed that an antisense oligonucleotide complementary to the Shine-Delagarno ribosomal docking sequence of *E. coli* 16S rRNA, inhibited translation of bacterial mRNA in cell-free extracts derived from *E. coli* (Jayaraman (1996) *Proc. Natl. Acad. Sci (USA)* 93:709-713). Furthermore, experiments were conducted by Gasparro et al. using a photoactivatable antisense DNA construct to suppress ampicillin resistance in *E. coli* (Gasparro et al. *Antisense Res. Dev.* (1991) 1:117-140). More recently, experiments utilizing antisense oligodeoxyribonucleotide phosphorothioates have shown the successful inhibition of the growth of a wild-type and drug resistant strain of *Mycobacterium smegmatis* (Rapaport et al. (1996) *Proc. Natl. Acad. Sci. (USA)* 93:709-713).

Bacteria have been known to mutate extensively, resulting in a large number of strains which have become resistant to most drugs presently available. In addition, new resistant bacterial strains are likely to develop as the time progresses. Thus, there is a continued need for development of additional therapeutic agents and effective methods to treat these bacterial infections. Inactivation or suppression of the multiple antibiotic resistance operon would ideally make some prokaryotes more susceptible to a larger number of antimicrobial agents and environmental stresses, thus providing novel means to counter increased bacterial resistance.

20 SUMMARY OF THE INVENTION

The invention disclosed herein satisfies this need. The present inventors have discovered that antisense oligonucleotides that are complementary to sequences found in the *marORAB* operon can inhibit or suppress the expression of one or more genes within that operon, thereby making bacteria more susceptible to a larger number of antimicrobial agents and environmental stresses.

This discovery has been exploited to develop the present invention which includes antisense oligonucleotides directed to mRNA derived from the *marORAB* operon sequences which specifically inhibit or suppress the expression of one or more genes within that operon, and which are therefore useful both as therapeutic agents and as tools to elucidate the role and biological significance of the *marORAB* operon sequences.

In one aspect, the invention provides a synthetic oligonucleotide complementary to a transcript of the *marORAB* operon which inhibits the expression of a gene in the operon.

As used herein, the term "operon" is a unit of bacterial gene expression and regulation. Typically an operon includes nucleic acid and control elements in the nucleic acid which may be recognized by regulators of gene products. In the case of

- 4 -

marORAB operon, the nucleic acid includes a regulatory region, designated *marO*, containing a promoter and an AUG start codon for rightward transcription of the loci designated *marR*, *marA* and *marB*.

For purposes of the invention, the term "transcript" is used to refer to ribonucleic acid transcribed from DNA, some of which is capable of serving as a substrate for the translation of one or more peptide products.

As used herein, the term "locus" refers to a position on a chromosome at which nucleic acid encoding a particular gene or genes reside. The nucleic acid comprises a start codon and at least one codon encoding an amino acid residue. Typically, a locus is transcribed to produce at least one mRNA transcript which in turn may be translated into a peptide.

As used herein, the term "synthetic oligonucleotide" refers to chemically synthesized polymers of nucleotides covalently attached by internucleotide linkages. The term "internucleotide linkage" is used to refer to the covalent bonding between nucleotides which are thus attached via at least one 5' to 3' internucleotide linkage.

In some embodiments of the invention the oligonucleotide contains at least one internucleotide linkage selected from the group consisting of alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. In some other embodiments, the oligonucleotide contains, in addition to an internucleotide linkage selected from the linkages recited, at least one phosphothioate internucleotide linkage.

In some embodiments, the oligonucleotides contain at least one deoxyribonucleotide, at least a ribonucleotide, or both deoxyribonucleotide(s) and ribonucleotide(s).

In some embodiments, the synthetic oligonucleotide is from about 15 to about 50 nucleotides in length. In preferred embodiments, these oligonucleotides contain from about 15 to about 21 nucleotides.

In some particular embodiments of the invention, *marORAB* oligonucleotides have a nucleotide sequence selected from the group consisting of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, and NO:6.

Another aspect of the invention provides a method of inhibiting the expression of the *marORAB* operon comprising the step of contacting a transcript of *marORAB* operon with a synthetic oligonucleotide complementary to the transcript. In some embodiments of the invention, the oligonucleotide is complementary to a locus selected from the group consisting of *marO*, *marO/R*, *marR*, *marR/A*, and *marA*. In yet other embodiments of the invention, the oligonucleotide comprises a nucleotide sequence

selected from the group consisting of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, and NO:6.

An additional aspect of the invention provides a method of reducing bacterial resistance to an antibiotic by exposing a resistant bacterium to synthetic oligonucleotide
5 complementary to a transcript of the *marORAB* operon. The bacterium further may be treated with an antibiotic.

The term "antibiotic" is art recognized and includes a composition which decreases the viability or which inhibits the growth or reproduction of microorganisms. As used in this disclosure, an antibiotic is further intended to include an antimicrobial or
10 bactericidal agent.

In an additional embodiment of the invention the oligonucleotide is complementary to a transcript of a locus selected from the group consisting of *marO*, *marO/R*, *marR*, *marR/A*, and *marA*. In yet another embodiment of the invention, the oligonucleotide comprises a nucleotide sequence selected from the group consisting of
15 SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, and NO:6.

In an additional embodiment, bacteria are contacted with at least two synthetic oligonucleotides selected from the group consisting of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, and NO:6.

Another aspect of the invention pertains to a method of treating a bacterial
20 infection in a subject by administering to the subject a therapeutic amount of a synthetic oligonucleotide complementary to a transcript of the *marORAB* operon which is effective in reducing bacterial resistance to antibacterial agent.

In one embodiment of the invention, the oligonucleotide is complementary to a transcript of a gene or locus selected from the group consisting of *marO*, *marO/R*, *marR*,
25 *marR/A*, and *marA*. In an additional embodiment of the invention the oligonucleotide includes a nucleotide sequence selected from the group consisting of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, and NO:6.

An additional aspect of the invention is a pharmaceutically acceptable composition comprising a synthetic oligonucleotide complementary to a transcript of
30 *marORAB* operon nucleic acid which inhibits the expression of one or more genes in the operon, and a physiologically acceptable carrier.

As used herein, the term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The term "physiologically acceptable" refers to a non-toxic
35 material that is compatible with a biological system such as a cell, cell culture, tissue, or organism.

- 6 -

In one embodiment of the invention, the oligonucleotide is complementary to a locus selected from the group consisting of *marO*, *marO/R*, *marR*, *marR/A*, and *marA*.

In yet another embodiment, the oligonucleotide consists essentially of SEQ ID NO:1, NO:2, NO:3, NO:4, NO:5, and NO:6.

5

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

10 FIG. 1 is a schematic representation of the *marORAB* operon, including the approximate location of sequences targeted by representative oligonucleotides of the invention; and

FIG. 2 is a graphic representation of the ability of representative antisense oligonucleotides of the invention to inhibit the expression of a *marA::lacZ* fusion in *E. coli*. The representative oligonucleotides are identified on the x axis, and the
15 corresponding spectrophotometric O.D. unit levels of β -galactosidase activities observed as a percent of the control in the absence of oligonucleotides are represented on the y axis.

20 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides antisense oligonucleotides specific for *marORAB* operon nucleic acid which are useful in treating diseases and disorders associated with drug resistance in prokaryotes. Antisense oligonucleotides of the invention are also useful for determining the role of *marORAB* sequences in pathogenicity, and more
25 specifically, in processes where drug resistance is involved.

MarORAB sequences have been reported in *E. coli* (George et al.(1983) *J. Bacteriol. supra*) in *Salmonella*, *Shigella*, *Klebsiella*, *Citrobacter*, *Hafnia*, and *Enterobacter* bacterial species (Cohen et al. (1993) *J. Infect. Dis.* 168:484-488). In addition, DNA-relatedness studies suggest that enteric bacteria in which the *marORAB*
30 regulatory operon was found to be conserved may be only a fraction of those in which *marORAB*-like sequences may be present. (Cohen et al. *ibid.*).

The *marORAB* locus at 34 min (1,636 kbp) on the *E. coli* chromosome map has been cloned and sequenced, and its regulation has been studied. (Cohen et al. *ibid.*; and Hachler et al. (1991) *J. Bacteriol.* 173:5532-5538). As shown in FIG. 1, the operon
35 includes a regulatory region, designated *marO*, containing a promoter-operator region for the rightward transcription of the *marR*, *marA* and the *marB* genes as well as the AUG start codon. The proteins encoded by these genes are MarR, a repressor protein

(Cohen et al. *ibid.*), MarA, a positive regulator protein whose overexpression leads to multidrug resistance, and MarB, a small protein which is required for the full resistance phenotype but whose function is yet unknown (George *J. Bacteriol. supra*; Yan et al. *Abstr. 1992 Gen. Meet. Am. Soc. Microbiol.* A-26, p.5).

5 The oligonucleotides of the invention are directed to any portion of the *marORAB* operon nucleic acid sequence that effectively acts as a target for inhibiting the expression of the genes within the *marORAB* operon.

FIG. 1 shows some non-limiting regions of the operon to which oligonucleotide of the invention may be directed. The nucleotide sequences of some representative,
10 non-limiting oligonucleotides specific for the *marORAB* operon are listed below in TABLE 1.

One of skill in the art, knowing the nucleotide sequence of the *marORAB* operon (Cohen et al. (1993) *J. Bacteriol. supra*) could prepare other oligonucleotides directed to these regions that inhibit the expression of one or more genes in the operon. For
15 example, other sequences targeted specifically to *marORAB* nucleic acid can be selected based on their ability to be cleaved by RNase H.

Preferred antisense oligonucleotides useful in the practice of the invention and suitable for use in therapeutic compositions of the invention are particularly active in inhibiting the expression of one or more genes in the *marORAB* operon. As used herein,
20 the term oligonucleotide includes polymers of two or more ribonucleotides, deoxyribonucleotides, 2' substituted ribonucleotides or deoxyribonucleotides or any combinations of monomers thereof, such monomers being connected together via 5' to 3' linkages which may include any of the linkages that are known in the antisense oligonucleotide art.

25 The term oligonucleotide also encompasses such polymers having chemically modified bases or sugars and/or having additional substituents including without limitation, lipophilic groups, intercalating agents, diamines adamantane and others. For example, oligonucleotides used in accordance with the invention may comprise other than phosphodiester internucleotide linkages between the 5' end of one nucleotide and
30 the 3' end of another nucleotide in which the 5' nucleotide phosphate has been replaced with any number of chemical groups, such as a phosphorothioate. The phosphorothioate linkages may be mixed Rp and Sp enantiomers, or they may be stereoregular or substantially stereoregular in either Rp or Sp form (see Iyer et al. (1995) *Tetrahedron Asymmetry* 6:1051-1054).

TABLE 1

5	TARGETED		SEQUENCE (5' to 3')	SEQ ID NO:
	OLIGO	SITE		
	92	<i>marA</i>	GCGTCTGGACATCGTCAT	1
	93	<i>marA</i>	ATCGTCATAGCTCTT	2
	1281	<i>marOR</i>	CTTTTCACATTAGTTGCCC	3
10	1282	<i>marR</i>	GCGCTTGTCATTCGGGTTC	4
	1283	<i>marO</i>	GTAATTAGTTGCAGAGGATA	5
	1284	<i>marO</i>	TAGTTGCAGGAGATAATATTG	6

15 Oligonucleotides with phosphorothioate linkages can be prepared using methods well known in the field such as phosphoramidite (see, e.g., Agrawal et al. (1988) *Proc. Natl. Acad. Sci. (USA)* **85**:7079-7083) or by H-phosphonate (see, e.g., Froehler (1986) *Tetrahedron Lett.* **27**:5575-5578) chemistry. The synthetic methods described in Bergot et al. (*J. Chromatog.* (1992) **559**:35-42) can also be used. Examples of other chemical

20 groups include alkylphosphonates, phosphorodithioates, alkyl phosphonothioates, phosphoramidates, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters or any combinations thereof. For example, U.S. Patent No. 5,149,797 describes traditional chimeric oligonucleotides having a phosphorothioate core region interposed between methylphosphonate or phosphoramidate flanking

25 regions. PCT Application No. PCT US96/13371, filed on August 31, 1996, discloses "inverted" chimeric oligonucleotides comprising one or more nonionic oligonucleotide region (e.g. alkylphosphonate and/or phosphoramidate and/or phosphotriester internucleoside linkage) flanked by one or more region of oligonucleotide phosphorothioate. Various oligonucleotides with modified internucleotide linkages can

30 be prepared according to known methods (see, e.g., Goodchild (1990) *Bioconjugate Chem.* **2**:165-187; Agrawal et al., (1988) *Proc. Natl. Acad. Sci. (USA)* **85**:7079-7083; Uhlmann et al. (1990) *Chem. Rev.* **90**:534-583; and Agrawal et al. (1992) *Trends Biotechnol.* **10**:152-158.

35 Examples of modifications to sugars include modifications to the 2' position of the ribose moiety which include but are not limited to 2'-O-substituted with an -O- lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl, or allyl group having 2-6 carbon atoms wherein such -O-alkyl, aryl or allyl group may be

unsubstituted or may be substituted, (e.g., with halo, hydroxy, trifluoromethyl cyano, nitro acyl acyloxy, alkoxy, carboxy, carbalkoxyl, or amino groups), or with an amino, or halo group. None of these substitutions are intended to exclude the native 2'-hydroxyl group in the case of ribose or 2'-H- in the case of deoxyribose. PCT Publication No.

- 5 WO 94/02498 discloses traditional hybrid oligonucleotides having regions of 2'-O-substituted ribonucleotides flanking a DNA core region. PCT Application No. PCT US96/13371, filed on August 31, 1996, discloses an "inverted" hybrid oligonucleotide which includes an oligonucleotide comprising a 2'-O-substituted (or 2' OH, unsubstituted) RNA region which is in between two oligodeoxyribonucleotide regions, a
- 10 structure that "inverted relative to the "traditional" hybrid oligonucleotides.

- Other modifications include those which are internal or are at the end(s) of the oligonucleotide molecule and include additions to the molecule at the internucleoside phosphate linkages, such as cholesteryl or diamine compounds with varying numbers of carbon residues between the two amino groups, and terminal ribose, deoxyribose and
- 15 phosphate modifications which cleave, or crosslink to the opposite chains or to associated enzymes or other proteins which bind to the bacterial genome. Examples of such modified oligonucleotides include oligonucleotides with a modified base and/or sugar such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide having a sugar which, at one or both its 3' and 5' positions is attached to a chemical group other
- 20 than a hydroxyl or phosphate group (at its 3' or 5' position). Other modified oligonucleotides are capped with a nuclease resistance-conferring bulky substituent at their 3' and/or 5' end(s), or have a substitution in one or both nonbridging oxygens per nucleotide. Such modifications can be at some or all of the internucleoside linkages, as well as at either or both ends of the oligonucleotide and/or in the interior of the molecule
- 25 (reviewed in Agrawal et al. (1992) *Trends Biotechnol.* 10:152-158).

- Preferably, oligonucleotides used in accordance with the invention will have from about 7 to about 50 nucleotides, more preferably from about 12 to 35 nucleotides, e.g. 12 to about 30, and most preferably from about 15 to about 21 nucleotides. Such oligonucleotides are preferably complementary to at least a portion of the targeted
- 30 mRNA transcript of the *marORAB* operon such that the oligonucleotide is capable of hybridizing or otherwise associating with at least a portion of such mRNA transcript under physiological conditions. Hybridization is ordinarily the result of base-specific hydrogen bonding between complementary strands of mRNA transcript preferably to form Watson-Crick or Hoogsteen base pairs, although other modes of hydrogen
- 35 bonding, as well as base stacking can also lead to hybridization.

Without being limited to any theory or mechanism, it is generally believed that the activity of oligonucleotides used in accordance with this invention depends is on the

binding of the oligonucleotide to the target nucleic acid (e.g. to at least a portion of an mRNA transcript thereof), thus disrupting the function of the target, either by hybridization arrest or by destruction of target RNA by RNase H (the ability to activate RNase H when hybridized to RNA). Such hybridization under physiological conditions
5 is measured as a practical matter by observing interference with the function of the nucleic acid sequence.

Thus, a preferred oligonucleotide used in accordance with the invention is capable of forming a stable duplex (or triplex in the Hoogsteen pairing mechanism) with the target nucleic acid, activate RNase H thereby causing effective destruction of the
10 target RNA molecule transcript, and in addition is capable of resisting nucleolytic degradation (e.g. endonuclease and exonuclease activity) *in vivo*. A number of the modifications to oligonucleotides described above and others which are known in the art specifically and successfully address each of these preferred characteristics.

The synthetic antisense oligonucleotides of the invention in the form of a
15 therapeutic formulation are useful in treating diseases, and disorders, and conditions associated with drug resistance in prokaryotes. Such formulations include a physiologically and/or pharmaceutically acceptable carrier. The characteristics of the carrier will depend on the route of administration. Such a composition may contain, in addition to the synthetic oligonucleotide and carrier, diluents, fillers, salts, buffers,
20 stabilizers, solubilizers, and other materials well known in the art. The pharmaceutical composition of the invention may also contain other active factors and/or agents which enhance inhibition of the expression of *marORAB* operon sequences or which will reduce drug resistance in prokaryotes. For example, combinations of synthetic oligonucleotides, each of which is directed to transcripts from different regions of the
25 *marORAB* operon, may be used in the pharmaceutical compositions of the invention. The pharmaceutical composition of the invention may further contain penicillins, cephalosporins, aminoglycosides, sulfonamides, macrolides, tetracyclines, lincosides, quinolones, chloramphenicol, vencomycin, metronidazole, rifampin, isoniazid, fm-butylethambutol, spectinomycin, trimethoprim, sulfamethoxazole, and others.

30 Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with the synthetic oligonucleotide of the invention, or to minimize side-effects caused by the synthetic oligonucleotide of the invention.

The pharmaceutical composition of the invention may be in the form of a
35 liposome in which the synthetic oligonucleotides of the invention is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals,

or lamellar layers which are in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. One particularly useful lipid carrier is lipofectin. Preparation of such liposomal formulations is within the level
5 of skill in the art, as disclosed, for example, in U.S. Patent No. 4,235,871; U.S. Patent No. 4,501,728; U.S. Patent No. 4,837,028; and U.S. Patent No. 4,737,323. The pharmaceutical composition of the invention may further include compounds such as cyclodextrins and the like which enhance delivery of oligonucleotides into cells, as described by Habus et al. (*Bioconjug. Chem.* (1995) 6:327-331), or slow release
10 polymers.

As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, i.e., healing of conditions associated with bacterial drug resistance. When applied to an individual active ingredient,
15 administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

In practicing the method of treatment or use of the present invention, a therapeutically effective amount of one or more of the synthetic oligonucleotides of the
20 invention is administered to a subject afflicted with a disease, disorder or condition associated with bacterial drug resistance. The synthetic oligonucleotide of the invention may be administered in accordance with the method of the invention either alone or in combination with other known therapies. When co-administered with one or more other therapies, the synthetic oligonucleotide of the invention may be administered either
25 simultaneously with the other treatment(s), or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering the synthetic oligonucleotide of the invention in combination with the other therapy.

Administration of the synthetic oligonucleotide of the invention used in the pharmaceutical composition or to practice the method of the present invention can be
30 carried out in a variety of conventional ways, such as, for example, oral ingestion, inhalation, or cutaneous, subcutaneous, intramuscular, or intravenous injection.

When a therapeutically effective amount of synthetic oligonucleotide of the invention is administered orally, the synthetic oligonucleotide will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the
35 pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% synthetic oligonucleotide and preferably from about 25 to 90% synthetic

oligonucleotide. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other
5 saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of the synthetic oligonucleotide and preferably from about 1 to 50% synthetic oligonucleotide.

When a therapeutically effective amount of synthetic oligonucleotide of the
10 invention is administered by intravenous, subcutaneous, intramuscular, intraocular, or intraperitoneal injection, the synthetic oligonucleotide will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous,
15 subcutaneous, intramuscular, intraperitoneal, or intraocular injection should contain, in addition to the synthetic oligonucleotide, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers,
20 preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

The amount of synthetic oligonucleotide in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of synthetic oligonucleotide
25 with which to treat each individual patient. Initially, the attending physician will administer low doses of the synthetic oligonucleotide and observe the patient's response. Larger doses of synthetic oligonucleotide may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. It is contemplated that the dosages of the pharmaceutical compositions
30 administered in the method of the present invention should contain about 0.1 to 5.0 mg/kg body weight per day, and preferably 0.1 to 2.0 mg/kg body weight per day. When administered systemically, the therapeutic composition is preferably administered at a sufficient dosage to attain a blood level of oligonucleotide from about 0.01 μM to about 10 μM .

35 The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. Ultimately

- 13 -

the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention. Some diseases lend themselves to acute treatment while others require longer term therapy.

- Antisense oligonucleotides of the invention specific for the *marORAB* operon
- 5 sequences are useful in determining the role of these sequences in pathogenicity, and more specifically in processes in which drug resistance is involved. For example, the efficacy of antisense technology in inhibiting bacterial drug resistance was measured in comparison to that of the wild type repressor *marR*. To measure the inhibitory effect of wild type *MarR*, DNA vectors containing *marR* sequences were introduced and
- 10 expressed in two different fusion cell lines containing *lac Z* sequences under the control of the *marORAB* operon. The cell lines used are wild type AG 100 (George et al. (1983), *supra*) and the *Mar* mutant AG 102. Wild type repressor *MarR* reduced *lacZ* expression from the 2 different fusions by 9-47 fold depending on the bacterial strain assayed.
- 15 Subsequently, to test the feasibility of using antisense technology to inhibit the expression of sequences under the control of the *marORAB* operon, a DNA vector containing both the *marR* and the *marA* genes cloned in the antisense direction (pKMN23), was tested for its ability to inhibit the production of β -galactosidase activity from *marA-lacZ* translational fusions. More specifically, pKMN23 was tested in
- 20 KMN14 (*marO*RA-lacZ*) and in KMN18 (*marORA-lacZ*) cells. Both KMN14 and KMN18 are translational fusion cell lines containing *marA* sequences in the same translational fusion as *LacZ*. KMN14 is a mutant containing a *mar-R2* mutation in the *marR* region. The results are shown in TABLE 2.

- 14 -

TABLE 2

STRAIN	GENE TRANSIENTLY EXPRESSED	β -GAL ACTIVITY	FOLD REDUCTION
<hr/>			
KMN14 (<i>marO*RA-lacZ</i>)		5130	-----
	<i>marR</i>	550	9.3
	mutant <i>marR</i>	4278	1.2
	<i>anti-marA</i>	950	5.4
KMN18 (<i>marORA-lacZ</i>)	-----	2741	-----
	<i>marR</i>	59	46.5
	mutant <i>marR</i>	1659	1.7
	<i>anti-marA</i>	56	48.9

As shown above, plasmids containing antisense *marA* sequences effectively reduced *LacZ* expression from the fusions by 5-49 fold, thus achieving an inhibitory effect comparable to that observed when adding wild type repressor MarR.

To establish further the inhibitory efficacy of antisense *marA* sequences, KMN23 (the same DNA vector described above, containing both the *marR* and the *marA* genes cloned in the antisense direction) was also expressed in the presence of various antibiotics (tetracycline (Tet), chloramphenicol (Cml), ampicillin (Amp) and rifampicin (Rif). The effect of KMN23 was assessed by measuring cell growth inhibition by the gradient plate method as described in Example 9. The results are shown in TABLE 3.

- 15 -

TABLE 3MIC/(μ g/ml)

PLASMID	Tet	Cml	Amp	Rif
AG 102 <i>mar</i> mutant	12.5	34.2	17.7	8.8
AG 102 with <i>marR</i> ⁺ plasmid	3.7	3.3	5.5	1.1
AG 102 with antisense- <i>marA</i> plasmid pKMN23	8.3	16.4	15	2.6

5

As shown above, the antisense construct reduced resistance to various unrelated antibiotics, hence proving that antisense sequences directed to sequences associated with the *marORAB* operon provide an ideal tool to inhibit bacterial drug resistance.

To optimize the inhibitory effect of the antisense oligonucleotides, KMN18 cells (a *marORA-lacZ* fusion *E. coli* cell line described above) were incubated with increasing concentrations of antisense oligonucleotides (10-20 μ M). Transcription of *marA* sequences was induced by the addition of sodium salicylate. Samples were assayed for β -galactosidase activity at 60 minute intervals. As shown in FIG. 2 all oligonucleotides added at 20 μ M concentrations reduced *marA-lacZ* activity. Control oligonucleotide 101C (SEQ ID NO:7) showed no reduction in *marA-lacZ* activity. In addition, antisense oligonucleotides 92 (SEQ ID NO:1) and 1284 (SEQ ID NO:6) reduced *marA-lacZ* activity from a cell line constitutively expressing a *marORA-lacZ* fusion protein (KMN14) (data not shown).

In order to ascertain the effects of increasing the oligonucleotides of the invention on the bactericidal activity of norfloxacin, competent cells of ML308-225-C2 (an isolated Mar mutant *E. coli* strain) were treated with increasing concentrations of representative antisense oligonucleotide 92 (SEQ ID NO:1), or oligonucleotide 1284 (SEQ ID NO:6), or control oligonucleotide 1403 (SEQ ID NO:8), (scrambled sequence of oligonucleotide 1284). TABLE 4 summarizes the results pertaining to antisense oligonucleotide 1284 in duplicate experiments.

- 16 -

TABLE 4

NORFLOXACIN OLIGO 1284		% OF CONTROL CELLS	
(μ g/ml)	(μ M)		
0	0	100	100
0.5	0	71	57
0.5	20	43	54
0.5	40	42	58
0.5	100	27	46
0.5	200	25	38

As shown, the killing effect of norfloxacin against ML308-C2 was enhanced significantly by the addition of antisense oligonucleotide 1284 (SEQ ID NO:6).

In order to establish the effects of increasing oligonucleotide concentrations on the antibacterial activity of antibiotics, Mar mutant cells were treated with oligonucleotide 1284 (SEQ ID NO:6) at a final concentration of 20, 40, 100 and 200 mM in the presence of 0.5 mg/ml of norfloxacin for 1 hour. Cell growth in the presence of the various concentrations of oligonucleotide 1284 (SEQ ID NO:6) was compared to that observed in untreated cells. The results are shown in TABLE 5.

TABLE 5

	NORFLOXACIN mg/ml	[OLIGO 1284] mM	% OF CONTROL (untreated cells)
5	----	----	100
	0.5	----	65
	0.5	20	48
	0.5	40	50
10	0.5	100	36
	0.5	200	31

As shown above for oligonucleotide 1284 (SEQ ID NO.6), the antisense oligonucleotides of this invention enhance the antibactericidal effect of antibiotics such as norfloxacin.

To ascertain that the inhibitory effect observed was a specific antisense effect causing the repression of the RNA message from the *marORAB* operon, the effect of oligonucleotide 1284 (SEQ ID NO:6) on bacterial growth was measured following one or two hours oligonucleotide treatment. Bacterial growth was compared to that observed following treatment with the scrambled negative control oligonucleotide 1403 (SEQ ID NO:8). Concentrations of 40 and 100 μ M of antisense oligonucleotides 1284 and scrambled oligonucleotide 1403 were shown to cause no change in cell viability in the absence of antibiotic. These results demonstrate that the killing effect of oligonucleotide 1284 shown above in TABLE 4 is not the result of the presence of the oligonucleotide alone, but rather, is the result of the combined action of the antibactericidal agent and the *marORAB* oligonucleotide of the invention.

The following examples illustrate the preferred modes of making and practicing the present invention, but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar results.

EXAMPLE 1

Preparation of Oligonucleotides

Synthesis of the following phosphothioate oligonucleotides: 92 (SEQ ID NO:1),
5 93 (SEQ ID NO:2), 1281 (SEQ ID NO:3), 1282 (SEQ ID NO:4), 1283 (SEQ ID NO:5),
1284 (SEQ ID NO:6), 101C (SEQ ID NO:7), 1403 (SEQ ID NO:8), RK1, (SEQ ID
NO:9), A2, (SEQ ID NO:10), LZ, (SEQ ID NO:11), ORAB2, (SEQ ID NO:12), and
RK3, (SEQ ID NO:13) was performed on a synthesizer (Pharmacia Gene Assembler
Series Synthesizer, Pharmacia LKB Biotechnology, Uppsala, Sweden) using standard b-
10 cyanoethylphosphoramidite procedure (see, Sonveaux "Protecting Groups in
Oligonucleotides Synthesis" in Agrawal (1994) *Methods in Molecular Biology* 26:1-72;
see also Uhlmann et al. (1990) *Chem. Rev.* 90:543-583). Agrawal) and amidites
(Cruachem, Glasgow, Scotland) and supports (Millipore, Bedford, MA). The random
20-mer phosphodiester contained an equimolar mixture of A, C, G, and T at each
15 position. DNAs were deprotected by treatment of the support with 1 ml of aqueous
NaOH at 55^o C for 16 hours. Subsequently the ammonia was removed from the
support, the support was washed with 200 ml of water, and the two fractions were
pooled and lyophilized. Following assembly and deprotection, the oligonucleotides
were ethanol precipitated twice, dried, and suspended in phosphate-buffered saline
20 (PBS) at the desired concentration.

The purity of these oligonucleotides was tested by capillary gel electrophoreses
and ion exchange HPLC. Endotoxin levels in the oligonucleotide preparation was
determined using the Luminous Amebocyte Assay (Bang (1953) *Biol. Bull.* (Woods
Hole, MA) 105:361-362).

25

EXAMPLE 2

Preparation of Constructs

A. Selection of a Mar Mutant of ML308-225

30 Antibiotic resistant mutants of ML308-225 (Rahman et al. (1991) *Antisense Res.*
Dev. 1:319-327) were grown and maintained at 30^oC in LB broth. These mutants were
selected by spreading washed overnight cultures onto a LB agar plate containing
chloramphenicol (5 µg/ml, Sigma, St. Louis, MO) and incubated at 30^oC for 48-72
hours. Single colonies appearing after 48 hours were picked for further study. Resistant
35 colonies were observed for multiple antibiotic resistance using antibiotic gradient plates.

One mutant showing increased minimal inhibitory concentrations (hereinafter
referred to as MIC) to tetracycline, chloramphenicol, ampicillin, nalidixic acid, and

- 19 -

norfloxacin was selected and designated ML308-C2. Sequencing confirmed a C @ T substitution in amino acid 117 of *marR*, which results in a truncated MarR protein. When wild type *marR* (*pMAL-marR*) was cloned into ML308-C2, the MIC's decreased to that of levels expressed by the wild-type ML308-225 strain (unpublished data).

- 5 Northern analysis using a *marA* DNA probe confirmed that ML308-C2 over expresses *marA* and was a Mar mutant (unpublished data). These results, in conjunction with the sequence data, support the conclusion that a *marR* mutation is responsible for the Mar phenotype in the ML308-C2 strain.

10 B. Construction of pDW10 and pDW11

- Plasmid DNA was prepared using the Promega WizardTM Prep Kit (Madison, WI). Restriction endonucleases and T4 DNA ligase (New England Biolabs, Beverly, MA) were used under conditions suggested by the supplier. PCR amplification was carried out using the Perkin Elmer Cetus DNA thermal cycler 480. *Taq Polymerase* and reagents (Perkin Elmer Cetus, Norwalk, CT) were used as directed to amplify the target sequence. Based on the known DNA sequence of the *mar* loci (GenBank accession #M96235), PCR primers were created which flanked the coding sequence and allowed amplification of *marA* and the *marORAB* operon region, which were then cloned behind the T7 promoter of pBLUESCRIPT KS (Stratagene, La Jolla, CA). The *marA* PCR primers were designed to amplify the *marA* coding sequence from 1893-2282 bp, resulting in a 389 bp product. A *marORAB* PCR product (1281 bp) was created as well using primers designed to amplify the DNA sequence of *marORAB* from 1311-2592 bp from the published sequence. Restriction endonuclease sites for *EcoRI* and *PstI* were incorporated into the ends of PCR primers to ensure that insertion of fragments were in the correct orientation when cloned into pBLUESCRIPT. pBLUESCRIPT-*marA* was termed pDW10 and pBLUESCRIPT-*marORAB* was named pDW11. To ensure that the proper DNA fragments were cloned, the DNA sequence of the cloned PCR products was determined by the method of Sanger et al. (*Proc. Natl. Acad. Sci. USA* (1977) 74:5463-5467) using the SequenaseTM sequencing kit (U.S. Biochemical, Cleveland, OH).

30

EXAMPLE 3**Transcription of Target RNA**

Ten µg each of the plasmids pDW10 (*marA*) and pDW11 (*marORAB*) were
5 linearized with *HindIII* (New England Biolabs, Beverly, MA) or *EcoRI* (New England
Biolabs, Beverly, MA) in a 50 µl reaction containing a 1X dilution of the appropriate
buffer supplied and 100 U of restriction enzyme. Cleavage was allowed to proceed at
37°C for 2 hrs. After this time the reaction was extracted twice with buffered
phenol/CHCl₃ (1:1), twice with CHCl₃/isoamyl alcohol (24:1), and precipitated with 3
10 volumes of precipitation mix (66 mM sodium acetate in ethanol). The DNA was
collected by centrifugation in a microfuge for 20 min, washed with 70% ethanol, and
dried briefly under vacuum. The resultant pellet was resuspended in 20 µl of water and
stored frozen until needed.

RNA bearing a hydroxyl group was transcribed from the linearized plasmids in a
15 20 µl reaction containing 1X supplied buffer, 1 mM each of rATP, rCTP, rUTP, 0.9 mM
guanosine, 0.1 mM GTP, 2 U/ml RNasin (Promega, Madison, WI), 2 µg linearized
plasmid, and 2.5 µl of 50,000 U/ml T7 RNA polymerase (New England Biolabs,
Beverly, MA), added in the order indicated. This reaction was incubated at 37°C for 1
hr and 1 unit of RQ1 DNase (Promega, Madison, WI) was added. DNA was allowed to
20 digest at 37°C for 15 min and then the RNA was purified using a ProbeQuant G-50 spin
column (Pharmacia Biotech, Piscataway, NJ) and the manufacturer's protocol. This
produced about 50 µl of solution containing the *marORAB* transcript. At this point, the
integrity of an aliquot of the transcript was checked by electrophoresis on a 1% agarose
TBE gel and ethidium bromide staining.

25

EXAMPLE 4**Radiolabelling of Target RNA**

The transcripts were radiolabelled with ³²P in a 20 µl reaction containing 10 µl
30 of transcript (1/5th of transcription), 2 µl of supplied T4 polynucleotide kinase buffer, 5
µl of γ-³²P-ATP (10 mCi/ml, Amersham, Cleveland, OH), 1 µl of 40 U/ml RNasin, and
2 µl of 10 U/µl T4 polynucleotide kinase (New England Biolabs, Beverly, MA). After 1
hr of incubation at 37°C, the transcript was purified using a spin column as described
above, yielding the radiolabelled transcript in a volume of approximately 50 µl.
35 Integrity of the transcript was checked by denaturing polyacrylamide electrophoresis and
autoradiography.

- 21 -

EXAMPLE 5**RNase H Mapping of Accessible Sites in Target RNA**

Mapping of sites accessible to oligonucleotide binding using RNase H as a probe for RNA/DNA duplex was done in a 10 µl reaction containing 5 µl of radiolabelled transcript, 1 µl of 10X RNase H buffer (400 mM Tris-HCl, pH 7.4, 40 mM MgCl₂, 10 mM dithiothreitol), 0.5 ml of 40 U/µl RNasin, 1 µl of 500 mM random 20-mer (heated and snap cooled), and 1.5 µl of water. This mixture was incubated at room temperature for 90 min and 1 µl of 1 U/ml RNase H (Boehringer Mannheim, Indianapolis, IN) was added. This constitutes the complete reaction. Control reactions lacking either random 20-mer, RNase H, or both were done in parallel. After 10 min at room temperature, the reaction was quenched by the addition of 10 µl of formamide loading dye. Samples were denatured by heating to 95°C for 5 min and 7 µl were analyzed by electrophoresis on a 4% polyacrylamide denaturing gel. Accessible sites were identified as abundant radiolabelled fragments unique to the complete reaction lane. Lengths of RNA fragments produced were calculated by comparison to a radiolabelled DNA restriction ladder. This ladder had been previously calibrated against RNAs of known lengths.

Sites accessible to oligonucleotide binding were found in both the 5' untranslated region (bases 1401-1450) and the coding region (bases 1708-1727) of *MarR* as well as near the translational start (bases 1890-1950) and in the coding region (bases 2040-2075) of *MarA* (numbering as in Gasparro et al. (1991) *Antisense Res. Dev.* 1:117-140).

EXAMPLE 6**Construction of Chromosomal*****marA-lacZ* and *marOR-lacZ* Fusions**

A translational fusion plasmid (pMLB1034) and a transcriptional fusion plasmid (pMBL1109) were used for constructing *marA-lacZ* fusions. A 818 bp *DraI* fragment, containing *marOR* and 144 bp of *marA*, were cloned into the *SmaI* site of the fusion plasmids. The resulting plasmids, pKMN14 and pKMN18 have the *marA* fusion in the same translation frame with *lacZ*. Plasmids pKMN19 and pKMN21 have *marA* inserted in a position upstream of a promoterless *lacZ* gene, thereby creating a transcriptional fusion. The *marOR(A)* fragment of pKMN14 and pKMN19 were derived from the pHHM191 plasmid which has a missense mutation at the 45th amino acid of *marR*, while pKMN18 and pKMN21 contained *marOR(A)* from the wild type pHHM183 plasmid. A 560 bp fragment bearing the *XmnI* site in *marR* to the *DraI* site in *marA* was

- 22 -

also inserted into the fusion plasmids leading to pKMN16, a *marA-lacZ* translational fusion plasmid and pKMN21, a *marA-lacZ* transcriptional fusion plasmid.

The *marOR*^{*}*A-lacZ*, *marORA-lacZ*, and *mar(R)A-lacZ* translational fusions in plasmids pKMN14, pKMN18, and pKMN16 respectively were transduced via a site
 5 specific recombination mechanism into the chromosome of ASS111, which contains a 1.24 kb *marORAB* deletion and is *lacZ*⁻, *phoA*⁻, and *recA*⁻. First, the fusion plasmids were transformed individually into ASS110, a *recA*⁺ strain (Seone, A. et al. (1996) *J. Bacteriol.* 177:530-535). The transformants were infected with λRZ5 to allow the formation of λRZ5(*marA-lacZ*) recombinants. The recombinant lysate was used to
 10 transduce the plasmid less ASS110 strain and ampicillin resistant (50 µg/ml) lysogens were selected. Lysates from these purified lysogens were then used to infect ASS111.

The presence of the *marA-lacZ* fusion in the chromosomal DNA of ampicillin resistant lysogenies was confirmed by PCR for the *marR-lacZ* fragment and the *marA-lacZ* fragment. Primer RK1 (5'-GTGAAAAGTACCAGCGATCTG-3'; SEQ ID NO:9),
 15 which can hybridize to the 5' terminal end of *marR* was used for the *marR-lacZ* fragment amplification and primer A2 (5'-GGTGAATTCATGACGATGTCCAGACGC-3'; SEQ ID NO:10), which can hybridize to the 5' terminal end of *marA*, was used for *marA-lacZ* fragment amplification. Primer LZ (5'-ATGTGCTGCAAGGCGAT-3'; SEQ ID NO:11), which can anneal to the internal portion of *lacZ*, was used as the *lacZ* primer in
 20 both constructions.

EXAMPLE 7

Cloning of *marR* and Antisense *marA*

Under *lac* and T7 Promoters

25 Primer ORAB2 (5'-GGACTGCAGGCTAGCCTTGCATCGCAT-3'; SEQ ID NO:12) hybridizes with nucleotides 1311 to 1328 in *marO* (Gen Bank sequence #M96235) and create a *PstI* site. Primer RK3 (5'-
 TCTTGAATTCTTACGGCAGGACTTTCTTAAG-3'; SEQ ID NO:13) hybridizes with
 30 nucleotides 1858 to 1879 at the 3' terminal end of *marR* and create an *EcoRI* site. These primers were used for amplification of the *marOR* gene from the wild type AG100 *E. coli* strain and the *Mar* mutant AG102 strain. The resulting 570 bp *PstI-EcoRI* PCR fragments were cloned into the *PstI-EcoRI* site of the pSPOK plasmid, a kanamycin resistant derivative of pSPORT1 (Gibco/BRL, Washington, D.C.), (Manneewannakul et
 35 al. (1994) *supra*). The *marOR*/pSPOK plasmid can be induced for the expression of the *marR* gene from either the *lac* or T7 promoter by IPTG or T7 RNA polymerase, respectively.

- 23 -

A 473 bp fragment from the *SacII* site in *marR* to the *DraI* site in *marA* was inserted into the *SnaBI* site of pSPOK. The resulting construct, pKMN23, in which the *marA* gene was directed in the opposite orientation of the *lac* promoter in pSPOK, was selected for the antisense *marA* construct. *MarA* was fused to *lacZ* in both transcriptional and translational fusions using cloning vectors pMLB1034 or pMLB1109). The fusions were introduced into a previously described *mar* deleted strain. Additionally, a 473 bp segment consisting of 330 bases of *marR* and 143 bases of *marA* was cloned in the antisense direction behind the *lac* promoter/T7 polymerase system (creating pKMN23).

EXAMPLE 8

DNA Transformation and Oligonucleotide Treatment of *E. coli*

DNA transformation into bacterial strains was performed using the CaCl_2 procedure as previously described by Cohen et al. *J. Bacteriol.* (1993) *supra*) or via electroporation. Competent cells (10^5) were transferred to tubes containing *marORAB* or control oligonucleotides in various concentrations. Oligonucleotide uptake was induced by a 1 minute heat shock (42°C) or through electroporation and the samples were incubated at 30°C for 1 or 2 hours. Depending on the experiment, strains incubated with the oligonucleotides were used in β -galactosidase assays, or colony formation and/or time kill experiments.

EXAMPLE 9

Gradient Plate Assay

Bacterial susceptibility to tetracycline hydrochloride, chloramphenicol, ampicillin, kanamycin, rifampin, nalidixic acid and norfloxacin was assayed by the gradient plate method. (*Microbiology: Including Immunology and Molecular Genetics* (3rd ed.)(Davis et al. eds.(1980)J.P. Lippincott Co., Philadelphia, PA).

- 24 -

EXAMPLE 10 **β -galactosidase Assays**

Strains KMN14 and KMN18, with or without antisense oligonucleotides, were grown at 30°C to mid-logarithmic phase. Fifty microliters of competent cells were treated with the appropriate oligonucleotide, heat shocked for 1 minute, and incubated at room temperature for 30 minutes to allow for annealing of the oligonucleotide with the mRNA. LB broth was then added to 1 ml, and the cell/oligonucleotide mixture was then incubated at 30°C for 30 minutes. After 30 minutes incubation, KMN18 (wild type) was induced with 5 mM sodium salicylate, whereas KMN14 was not (constitutively expressed *mar* mRNA). Samples of cell/oligonucleotide mixtures were then removed at 1 hour intervals and assayed for β -galactosidase the method of Miller (*Immunochemistry* (1972) 9:217-228).

EXAMPLE 11**Inhibition of β -galactosidase Activity in KMN18**

The *marA* transcript was inducible with 5 mM sodium salicylate after 30 minutes incubation in *E. coli* KMN18 strain (*a marORA-lacZ fusion cell line*). KMN18 was incubated with increasing concentrations (4-20 μ M) of antisense oligonucleotides. The cells were then induced with 5 mM salicylate. The samples were removed after 30 and 60 minute intervals and assayed for β -galactosidase activity. After 30 and 60 minute incubations with salicylate, oligonucleotides 92 and 1284 at 20 μ M concentrations caused reduced *marA-lacZ* activity (FIG. 2). Control Oligonucleotide 101 showed no reduction in *marA-lacZ* activity. At 20 μ M, the same and an additional oligonucleotide, 1403, a scrambled 1284, were tested. In four separate experiments oligonucleotides 92 and 1284 showed significant activity. These studies showed that antisense oligonucleotides 92 and 1284 showed a dose response effect on *LacZ* expression.

EXAMPLE 12**Augmentation of Bactericidal Activity of Norfloxacin Using *marORAB*-specific Oligonucleotides**

Competent cells of ML308-225-C2 (Mar mutant)(50 μ l, 10⁷ cells) were incubated with increasing concentrations of antisense oligonucleotides 1403, 92, or 1284 for 30 minutes on ice. The cell/oligonucleotide mixture was either heat shocked at 42°C for one minute or electroporated (to allow for uptake). The cell suspension was placed on ice for two minutes and then placed at room temperature for 30 minutes to allow for

- 25 -

association of the antisense oligonucleotide with the mRNA. LB broth (450 µl) was added to the mixture which was incubated for 1 and 2 hours at 30°C. After 30 minutes of incubation at 30°C, 1X the MIC of norfloxacin (0.5 µg/ml) was added to the cell suspension. At 1 hour post exposure to norfloxacin, 100 µl aliquots of

5 cell/oligonucleotide mixtures were collected, diluted into PBS and the various dilutions were plated on LB agar plates. Following overnight incubation at 30°C, the number of colonies was counted on all plates. For all experiments, the samples were plated in duplicate or triplicate and all colonies on a plate were counted.

Antisense oligonucleotide 1284 (SEQ ID NO:6) enhanced the killing effect of
10 norfloxacin against ML308-C2 as shown above in TABLE 4. In two separate experiments, there was a greater effect with 100-200 µM vs. 20-40 µM oligonucleotide. Oligonucleotide 1284 was then compared to its scrambled control 1403 (SEQ ID NO:8) to determine if its activity was a specific antisense effect directed at repressing the *marRAB* RNA message. Concentrations of 40 and 100 µM were chosen based on the
15 results of TABLE 4. As shown in TABLE 5 the presence of control Oligonucleotide 1403 alone caused no change in cell viability. At 1 hour post exposure to 1X MIC of norfloxacin, 1284 exhibited a 23% drop in viability as compared to 6% with 1403. This effect seemed to diminish at 2 hours. The results showed a specific effect of oligonucleotide 1284 in enhancing the killing effects of norfloxacin against a *mar*
20 mutant which had been resistant to the bactericidal effect of norfloxacin.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and
25 procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

- 26 -

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- 5
- (i) CO-APPLICANTS:
- (A) NAME: HYBRIDON, INC.
- (B) STREET: 620 MEMORIAL DRIVE
- 10 (C) CITY: CAMBRIDGE
- (D) STATE: MA
- (E) COUNTRY: US
- (F) POSTAL CODE (ZIP): 02139
- (G) TELEPHONE:
- 15 (H) TELEFAX:
- (A) NAME: TRUSTEES OF TUFTS COLLEGE
- (B) STREET: TUFTS UNIVERSITY, BALLOU HALL, 4TH FLOOR
- (C) CITY: MEDFORD
- 20 (D) STATE: MA
- (E) COUNTRY: US
- (F) POSTAL CODE: 02155
- (G) TELEPHONE:
- (H) TELEFAX:
- 25 (ii) TITLE OF INVENTION: OLIGONUCLEOTIDES SPECIFIC FOR
THE *marORAB* OPERON
- (iii) NUMBER OF SEQUENCES: 13
- 30 (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: LAHIVE & COCKFIELD, LLP
- (B) STREET: 28 State Street
- (C) CITY: Boston
- (D) STATE: Massachusetts
- 35 (E) COUNTRY: USA
- (F) ZIP: 02109
- (v) COMPUTER READABLE FORM:
- 40 (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
- 45 (A) APPLICATION NUMBER: PCT/US98/
- (B) FILING DATE:
- (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- 50 (A) APPLICATION NUMBER: USSN 60/038,663
- (B) FILING DATE: 21 FEBRUARY 1997
- (viii) ATTORNEY/AGENT INFORMATION:
- 55 (A) NAME: Hanley, Elizabeth A.
- (B) REGISTRATION NUMBER: 33,505

- 27 -

(C) REFERENCE/DOCKET NUMBER: PKZ-003PC

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(2) INFORMATION FOR SEQ ID NO:1:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGTCTGGAC ATCGTCAT

18

(2) INFORMATION FOR SEQ ID NO:2:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATCGTCATAG CTCTT

15

40 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
45 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

55 CTTTTTCACAT TAGTTGCCC

19

- 28 -

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

15 GCGCTTGTCA TTCGGGTTC

19

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

30

GTAATTAGTT GCAGAGGATA

20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

45

TAGTTGCAGG AGATAATATT G

21

(2) INFORMATION FOR SEQ ID NO:7:

50

(i) SEQUENCE CHARACTERISTICS:

- 55 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- 29 -

(ii) MOLECULE TYPE: cDNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTGACTGCCA ACTATGAACA

20

10 (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTTCAGAGTT AGTAGGAATA T

21

25 (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
30 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTGAAAAGTA CCAGCGATCT G

21

40 (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
45 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGTGAATTCA TGACGATGTC CAGACGC

27

- 30 -

(2) INFORMATION FOR SEQ ID NO:11:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- 10 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

15

.ATGTGCTGCA AGGCGAT

17

(2) INFORMATION FOR SEQ ID NO:12:

- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
25 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGACTGCAGG CTAGCCTTGC ATCGCAT

27

(2) INFORMATION FOR SEQ ID NO:13:

35

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
40 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TCTTGAATTC TTACGGCAGG ACTTTCTTAA G

31

- 31 -

What is claimed is:

1. A synthetic oligonucleotide complementary to a transcript of the *marORAB* operon which inhibits expression of the operon.
2. The oligonucleotide of claim 1 wherein the gene whose expression is inhibited is selected from the group consisting of *marO*, *marO/R*, *marR*, *marR/A*, and *marA*.
3. The oligonucleotide of claim 1 wherein the oligonucleotide contains at least one internucleotide linkage selected from the group consisting of alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters.
4. The oligonucleotide of claim 3 wherein the synthetic oligonucleotide contains at least one phosphorothioate internucleotide linkage.
5. The oligonucleotide of claim 1 comprising at least one deoxyribonucleotide.
6. The oligonucleotide of claim 1 comprising at least one ribonucleotide.
7. The oligonucleotide of claim 5 comprising at least one ribonucleotide.
8. The oligonucleotide of claim 1 wherein the oligonucleotide is from about 15 to 21 nucleotides in length.
9. The oligonucleotide of claim 1 having a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, NO:2, NO: 3, NO:4, NO: 5, and NO: 6.
10. A method of inhibiting expression of the *marORAB* operon comprising the step of contacting a transcript of the *marORAB* operon nucleic acid with a synthetic oligonucleotide complementary to the transcript.

- 32 -

11. The method of claim 10 wherein the oligonucleotide is complementary to a transcript of a gene selected from the group consisting of *marO*, *marO/R*, *marR*, *marR/A*, and *marA*.

5 12. The method of claim 11 wherein the oligonucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, NO: 2, NO: 3, NO: 4, NO: 5, and NO: 6.

10 13. A method of reducing bacterial resistance to an antibiotic comprising exposing a bacteria in a subject with an infection to a synthetic oligonucleotide complementary to a transcript of the *marORAB* operon such that bacterial resistance to an antibiotic is reduced.

15 14. The method of claim 13 wherein said oligonucleotide is administered to the subject in a pharmaceutical carrier.

15. The method of claim 14, comprising administering in addition at least one antibiotic to the subject.

20 16. The method of claim 15, wherein the antibiotic is selected from the group consisting of a penicillin, a cephalosporin, an aminoglycoside, a sulfonamide, a macrolide, a tetracycline, a lincoside, a quinolone, a chloramphenicol, a vancomycin, a rifampin, an isoniazid, or a trimethoprim.

25 17. The method of claim 13, wherein the oligonucleotide is complementary to a transcript of a locus selected from the group consisting of *marO*, *marO/R*, *marR*, *marR/A*, and *marA*.

30 18. The method of claim 13, wherein the oligonucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, NO: 2, NO: 3, NO: 4, NO: 5, and NO: 6.

35 19. The method of claim 18, wherein the bacteria are exposed to at least two synthetic oligonucleotides having a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, NO: 2, NO: 3, NO: 4, NO: 5, and NO: 6.

- 33 -

20. The method of claim 13, wherein the infection in the subject is a species of *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, *Citrobacter*, *Hafnia*, or *Enterobacter*.

21. A method of treatment for a bacterial infection in a subject by
5 administering to the subject a therapeutic amount of a synthetic oligonucleotide complementary to a transcript of the *marORAB* operon.

22. The method of claim 21, wherein the bacterial infection comprises infection with at least one bacterium having multiple drug resistance.

10

23. The method of claim 21 wherein the subject is additionally administered an antibiotic.

24. The method of claim 23, wherein the antibiotic is selected from the group
15 consisting of a penicillin, a cephalosporin, an aminoglycoside, a sulfonamide, a macrolide, a tetracycline, a lincoside, a quinolone, a chloramphenicol, a vancomycin, a rifampin, an isoniazid, or a trimethoprim.

25. The method of claim 21, wherein the infection comprises an
20 *Escherichia*, a *Salmonella*, a *Shigella*, a *Klebsiella*, a *Citrobacter*, a *Hafnia*, or an *Enterobacter*.

26. The method of claim 21 wherein the oligonucleotide is complementary to a transcript of a gene selected from the group consisting of *marO*, *marO/R*, *marR*,
25 *marR/A*, and *marA*.

27. The method of claim 26 wherein the oligonucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, NO: 2, NO: 3, NO: 4, NO: 5, and NO: 6.

30

28. A pharmaceutically acceptable composition comprising a synthetic oligonucleotide complementary to a transcript of *marORAB* operon which inhibits the expression of a locus in the operon; and a physiologically acceptable carrier.

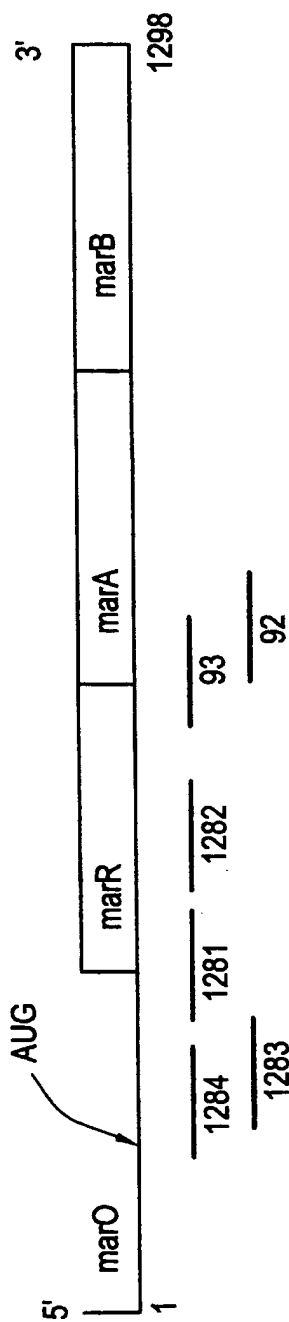
35 29. The pharmaceutically acceptable composition of claim 28 wherein the oligonucleotide is complementary to a transcript of a gene selected from the group consisting of *marO*, *marO/R*, *marR*, *marR/A*, and *marA*.

- 34 -

30. The pharmaceutically acceptable composition of claim 28 wherein the oligonucleotide has a nucleotide sequence consisting essentially of SEQ ID NO: 1, NO: 2, NO: 3, NO: 4, NO: 5, and NO: 6.

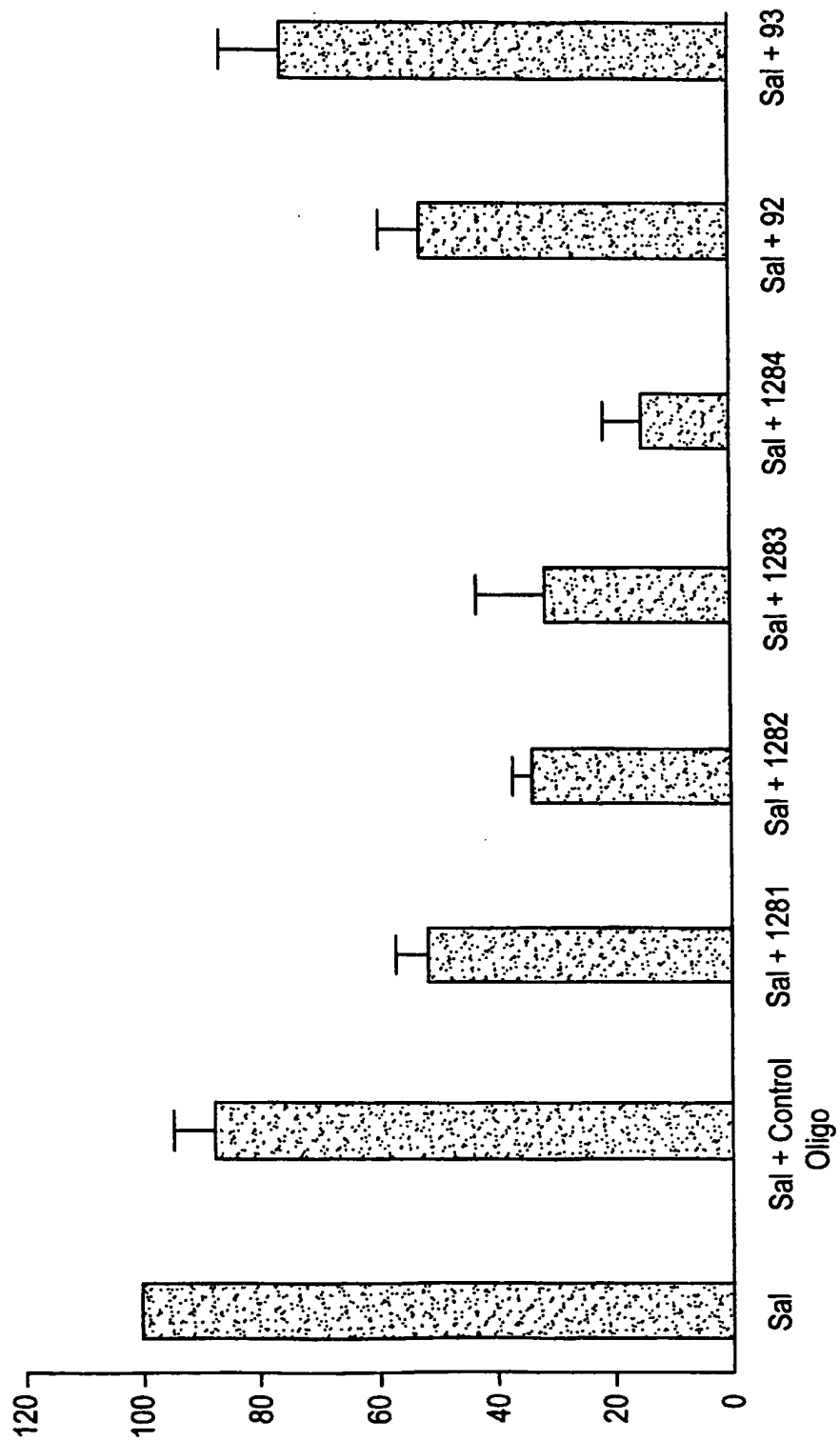
1 / 2

FIG. 1



2 / 2

FIG.2



INTERNATIONAL SEARCH REPORT

Intern. Patent Application No.

PCT/US 98/02999

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/11 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 05810 A (TUFTS COLLEGE) 17 March 1994	9,12,18, 19,27,30
Y	see the whole document	1,3-8, 10,11, 17,20,25
Y	<p>--- WO 93 13740 A (WORCESTER FOUND EX BIOLOGY) 22 July 1993 cited in the application see the whole document ---</p> <p style="text-align: center;">-/-</p>	3-8

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

1 July 1998

Date of mailing of the international search report

29.07.98

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 98/02999

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J.D. GOLDMAN ET AL., : "Multiple antibiotic resistance (mar) locus protects Escherichia coli from rapid cell killing by fluoroquinolones" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 40, no. 5, 1996, WASHINGTON, DC, US, pages 1266-1269, XP002069193 cited in the application see the whole document and specially table 2	1,10,11, 17,20,25
X	--- S.P. COHEN ET AL., : "Genetic and functional analysis of the multiple antibiotic resistance (mar) locus in Escherichia coli" JOURNAL OF BACTERIOLOGY, vol. 175, no. 5, 1993, WASHINGTON, DC, US, pages 1484-1492, XP002069168 cited in the application see abstract; figure 1	9,12,18, 19,27,30
X	--- L. GAMBINO ET AL.,: "Overexpression of the marA positive regulator is sufficient to confer multiple antibiotic resistance in Escherichia coli" JOURNAL OF BACTERIOLOGY, vol. 175, 1993, WASHINGTON, DC, US, pages 2888-2894, XP002069169 see the whole document and specially figures 2 and 3	9,12,18, 19,27,30
X	--- EMBL NUCLEOTIDE SEQUENCE DATABASE, 29 January 1997, HINXTON, GB, XP002069932 Accession number= AE000250. E. coli marR, marA and marB genes. -& F.R. BLATTNER ET AL., : "The complete genom sequence of Escherichia coli K-12" SCIENCE, vol. 277, 5 September 1997, WASHINGTON, DC, US, pages 1453-1462, XP002069950 see the whole document	9,12,18, 19,27,30
A	--- WO 94 02498 A (WORCESTER FOUND EX BIOLOGY) 3 February 1994 see the whole document	1,3-8, 10,11,17
A	--- EP 0 472 434 A (BAYLOR COLLEGE MEDICINE ;ABBOTT LAB) 26 February 1992 see the whole document	1,10,11, 17,20,25
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INTERNATIONAL SEARCH REPORT

Intern. Appl. Application No.

PCT/US 98/02999

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S.P. COHEN ET AL., : "A multidrug resistance regulatory chromosomal locus is widespread among enteric bacteria " THE JOURNAL OF INFECTIOUS DISEASES, vol. 168, no. 2, 1993, CHICAGO, ILL, US, pages 484-488, XP002069167 cited in the application see the whole document and specially table 1 ---	1,10,11, 17,20,25
A	S. AGRAWAL: "Antisense oligonucleotides as antiviral agents" TRENDS IN BIOTECHNOLOGY, vol. 10, no. 5, 1992, CAMBRIDGE, GB, pages 152-158, XP000272382 cited in the application see the whole document ---	3-8
A	G.D. HOKE ET AL., : "Effects of phosphorothioate capping on antisense oligonucleotide stability, hybridization and antiviral efficacy versus herpes simplex virus infection" NUCLEIC ACIDS RESEARCH, vol. 19, no. 20, 1991, OXFORD, GB, pages 5743-5748, XP002014768 see the whole document ---	3-8
A	V. METALEV ET AL., : "Study of antisense oligonucleotide phosphorothioates containig segments of oligodeoxynucleotides and 2'-O-methoxyloligoribonucleotides" BIOORGANIC AND MEDICAL CHEMISTRY LETTERS, vol. 4, no. 24, 1994, OXFORD, GB, pages 2929-2934, XP002020356 see the whole document ---	3-8
P,X	D.G. WHITE ET AL., : "Inhibition of multiple antibiotic resistance (mar) operon in Escherichia coli by antisense DNA analogs" ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 0, no. A-69, May 1997, WASHINGTON, DC, US, page 12 XP002069170 see abstract --- -/--	1-30

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 98/02999

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>D.G. WHITE ET AL., : "Inhibition of the multiple antibiotic resistance (mar) operon in Escherichia coli by antisense DNA analogs"</p> <p>ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 41, no. 12, 1 December 1997, WASHINGTON, DC, US, pages 2699-2704, XP002069171</p> <p>see the whole document</p> <p>-----</p>	1-30

INTERNATIONAL SEARCH REPORT

Int. application No.
PCT/US 98/02999

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 13-28 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/US 98/02999

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